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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The principle investigator has made substantial progress in executing the statement of work outlined in the proposal. In addition he has fulfilled the requirements mandated by his training program to date. First, the PI obtained the human C5 cDNA and substituted a PSA substrate sequence in place of the wild type activation sequence. Modifications were implemented based on results from homology modeling. Next, PSA mediated cleavage was confirmed by mass spectrometry. Recombinant production of the confirmed PSA cleavable C5 mutant was scaled up by an adenovirus for in vitro assays. Purification of the recombinant protein was achieved using His-chromatography. PSA-mediated cleavage of the mutant was analyzed by western blot which resulted in curious results. It was then determined wtC5 and wtC3 are substrates of PSA. Ongoing studies include reverse engineering wild-type C5 to make PAC-2 selectively activateable.

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Introduction:

The principle investigator (PI) has made substantial progress in fulfilling the goals outlined in the statement of work. Several major unforeseen challenges have been surmounted. Results that first appeared negative have been reanalyzed and may be a serendipitous discovery into the initiation and progression of prostate cancer. The PI has completed his thesis proposal and formed a five-member thesis committee that has since met five times. The PI assisted his mentor in the acquisition of additional funding. The PI has presented his research at three meetings.

Body:

Specific Aim 1 – Obtain human C5 cDNA and substitute a PSA substrate sequence in place of the wild type activation sequence; amend a purification tag to the terminus of C5 to aid in its purification. **[Completed]**

The C5 cDNA was mutated via site-directed mutagenesis to include a PSA recognition sequence in place of the wild type activation sequence. The resulting construct was named PAC-1. PAC-1 was transfected into COS-7 cells and the supernatant containing the recombinant protein was collected. Because of the chance the purification tag could alter the conformation and thus hinder the PSA mediated cleavage, we decided to add the tag on after cleavage was observed (in Specific Aim 2).

Specific Aim 2 – Verify PSA has the capacity to cleave PAC5. Write thesis proposal and form "Thesis Advisory Committee". **[Completed]**

The recombinant protein collected above was characterized for PSA mediated cleavage by western blot analysis. Briefly, purified human PSA (Calbiochem) was incubated at 10µg/mL in the conditioned media containing the recombinant protein. The protein solution was then analyzed for cleavage by western blot using a C5 antibody (Santa Cruz Biotechnology). Cleavage would be verified by a shift in molecular weight. Unfortunately, no shift was observed.

Following the guide of Ogata and Low[1], we made a series of mutants (Figure 1) incorporating more of the semenogelin II sequence, the known physiological substrate for PSA. In all, six more mutants were generated. Meanwhile, the mutants were examined *in silico* for subtle structural changes which may render them PSA sensitive. Briefly, homology modeling was performed using the SWISS-MODEL[2] homology modeling server via the automated mode using the published crystal structure of C5 as a template (PDB 3CU7)[3]. The homology models suggested adding six more residues of semenogelin II to the left of the PSA recognition sequence and seven more residues of semenogelin II to the right would result in a α-helix containing the "HSSKLQ" PSA substrate sequence being exposed to the solvent (Figure 2). This mutant was named PAC-2.

C5a — C5b (wild type)

C5a – HSSKLQ// – C5b

C5a – **VDVREE**HSSKLQ// – C5b

C5a – VDVREEHSSKLQ//**T** – C5b

C5a – VDVREEHSSKLQ//**TS** – C5b

C5a – VDVREEHSSKLQ//**TSLH** – C5b

C5a – VDVREEHSSKLQ//**TSLHP** – C5b

C5a – VDVREEHSSKLQ//**TSLHPAH** – C5b

Figure 1: Wild type C5 and a series of mutants incorporating the semenogelin II sequence. Briefly, the sequence connecting C5a and C5b was mutated to resemble the physiological substrate for PSA.

Figure 8. Example of PAC5 mutants that Will be generated based on PSA substrate HSSKLQ
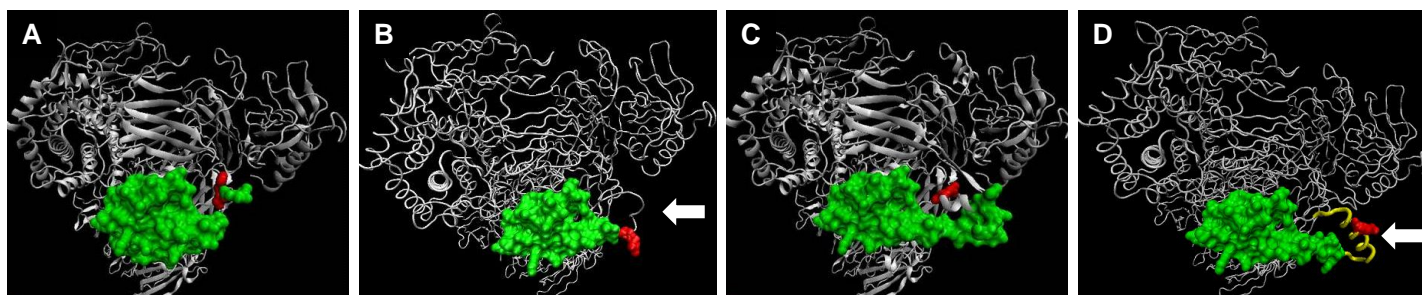
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Figure 2: Models of (a) wild type C5; (b) Mut-5 protein described by Ogata et al; (c) PAC5-1 mutant with HSSKLQ at the cleavage site; (d) PAC5-2 mutant with additional flanking sequences to create a loop that may be accessible to PSA cleavage. Amino acid at cleavage site depicted in red, N-terminal fragment in green, PAC5-2 insert in yellow.

A plasmid containing PAC-2 was transfected into COS-7 and HEK293T cells and conditioned media containing the recombinant protein was collected. For unknown reasons expression levels of PAC-2 were substantially lower in both cell lines. The recombinant PAC-2 was analyzed for PSA mediated cleavage by western blot as described above. Unfortunately, no shift was observed, although signal was weak and irreproducible because of low protein levels. We suspected some cleavage might actually be occurring, but at subprime levels. This combined with the small amount of PAC-2 protein made analysis by western blot problematic.

As a more sensitive way to probe for PSA mediated PAC-2 cleavage, we analyzed PSA treated conditioned media by MALDI-TOF mass spectroscopy. In this experiment, wild type C5 was completely resistant to cleavage by PSA and no release of 11 kDa fragment was observed (Figure 3). In contrast, incubation of the PAC5-2 protein resulted in production of an ~11kDa fragment corresponding to the modified N-terminal portion of the PAC5-2 protein. Based on the MALDI results, high resolution LTQ nanoHPLC/Orbitrap mass spectroscopic analysis was performed to obtain high resolution mass. Sequencing of this mass fragment using the Sequest sequencing algorithm confirmed the correct sequence corresponding to the 80 amino acid N-terminal C5 cleavage product
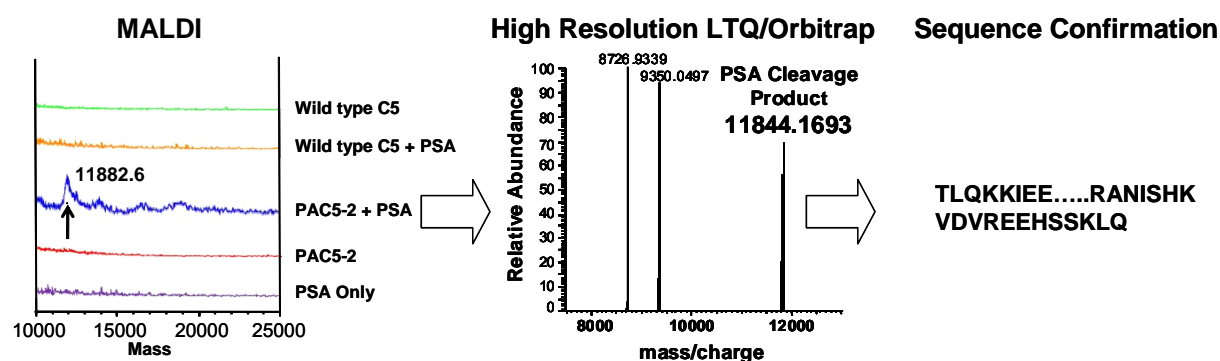

Figure 3: Comparison of PSA cleavage of wild type C5 vs. PAC5-2 mutant. Lower resolution MALDI-ToF demonstrates generation of 11kDa peak corresponding to N-terminal fragment released from C5. High resolution mass spectroscopy using LTQ/Orbitrap gives correct mass confirms the correct sequence.

Over this time period the PI wrote his thesis proposal and formed a thesis committee consisting of five faculty members at the Johns Hopkins School of Medicine:

Dr. R. Brodsky
Dr. S. Denmeade
Dr. S. Kachhap
Dr. S. Lupold
Dr. H. Wade

Specific Aim 3 – Demonstrate PAC5's selective toxicity to PSA producing prostate cancer cells but not PSA-null prostate cancer cells in presence of purified complement components C6-C9. ***[In progress]***

To demonstrate selective toxicity in a tissue culture model we first needed to scale up production of PAC-2. Transient expression resulted in very low yields of protein, so it was decided another expression system would have to be used. A recombinant adenovirus expressing PAC-2 was made as described by He et al[4]. Briefly, PAC-2 was cloned into pAdTrack-CMV. pAdTrack-CMV-PAC-2 was transformed into electrocompetent AdEasier cells already harboring pAdEasy-1. Homologous recombination occurs in the AdEasier cells resulting in a ~40kB adenovirus expressing the PAC-2 DNA. This DNA was purified and transfected into HEK293T cells. Virus production was monitored by GFP expression. After a series of viral amplifications a high titer virus stock was made.

Recombinant PAC-2 can be expressed by adding viral stock to feeder HEK293 cells. A series of viral transfections were performed to optimize the ratio of recombinant adenovirus to feeder cells. Unfortunately, it was soon realized that because of the replicative nature of the adenovirus we would have to discontinue the use of HEK293s. Viral transfection of the HEK293s not only results in the production of PAC-2, but also the production of more adenovirus, which in turn lyses the cells before maximum levels of PAC-2 can be achieved. Vero cells, isolated from the African green monkey, were chosen to express PAC-2. Because Vero cells do not harbor the genes required for the adenoviral life cycle, the cells make PAC-2 but not make more adenovirus and lyse the cells.

Two tissue culture flasks (each 75cm^2) were seeded with Vero cells and allowed to grow to 95% confluency. At this point 500μL of viral stock was added for infection. Four and a half hours later the media was aspirated and the monolayers were washed once with PBS. To ease downstream purification serum-free media was added. Transfection efficiency was monitored by GFP expression. Approximately 100hours later the conditioned media was harvested.

The conditioned media was subject to Ni-NTA batch and column purification and analyzed by western blot using an anti-penta-His antibody (figure 4). Acceptable purity was obtained, judged by western blot.
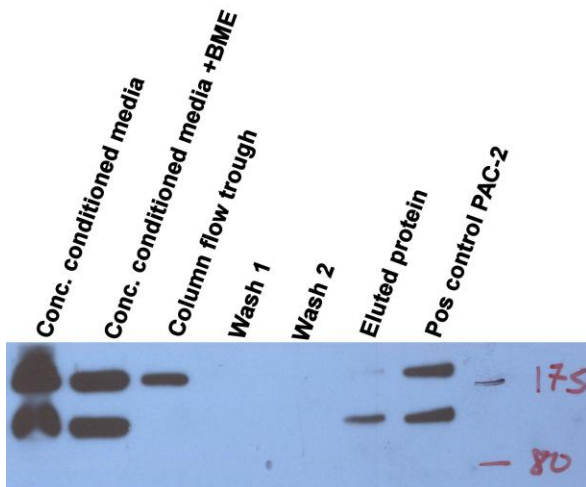

Figure 4: Western blot of concentrated conditioned media and His-purified PAC-2 using an anti-penta-His antibody. Concentrated conditioned media show two bands of PAC-2, one at ~190kDa, and another at 115kDa. It is likely the top band is unprocessed PAC-2 because of the addition of BME did not reduce the disulfide bond between the alpha and beta chains. However, for an unknown reason primarily the unprocessed PAC-2 flows though the column without binding, while the eluted protein is mostly processed PAC-2.

Figure 5: Results of PSA mediated PAC-2 cleavage, analyzed by western blot using an anti-penta-His antibody. PAC-2 was added to enzymatically active PSA in buffer or buffer alone. Time points were taken at 0 hour, 1 hour, 3 hours, and overnight. In absence of PSA, PAC-2 seems to be stable in buffer as no significant shifts were observed. However, when PAC-2 is treated with PSA both the unprocessed PAC-2 (190kDa) and processed PAC-2 (115kDa, alpha chain) show a decrease in levels, suggesting some sort of proteolysis is occurring.
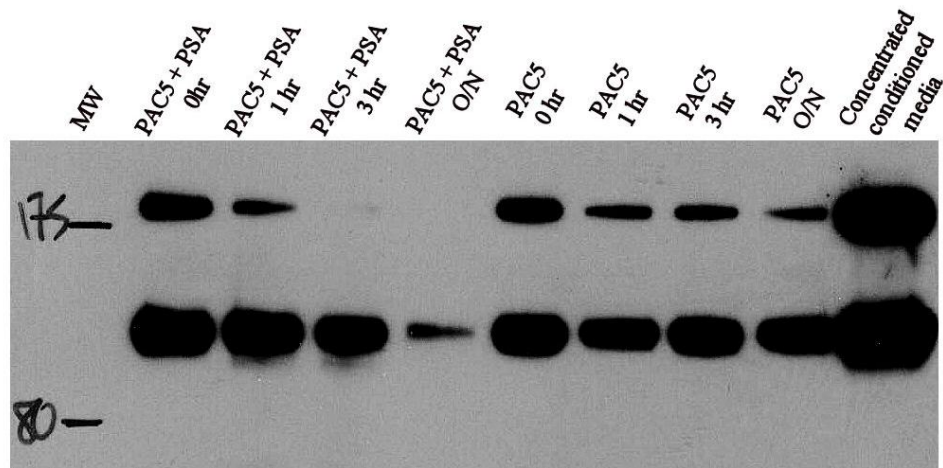
PSA mediated PAC-2 cleavage was carried out in PSA buffer and reactions were analyzed by western blot. We were pleased to see PSA-mediated cleavage of PAC-2 (figure 5), but were confused by the pattern of cleavage. We expected the alpha chain (115kDa) to shift down approximately 11kDa representing the liberation of the C5a fragment as observed by Ogata and Low[1]. Surprisingly, the alpha chain disappeared altogether.
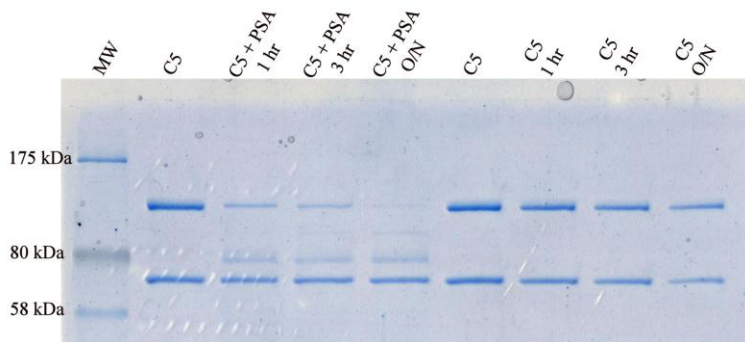

Figure 6: Results of PSA mediated wild-type C5 cleavage, analyzed by gel electrophoresis and protein staining. C5 was added to enzymatically active PSA in buffer or buffer alone. Time points were taken at 0 hour, 1 hour, 3 hours, and overnight. In the absence of PSA, C5 seems to be stable in buffer as no significant shifts were observed. However, when C5 is treated with PSA the 115kDa alpha chain appears to be cleaved twice.

As a negative control we choose to repeat the experiment with wild-type C5 to ensure it was not cleaved by PSA. In a similar manner as PAC-2 we treated C5 with PSA and looked for the presence of proteolytic products. We were surprised to learn PSA was able to cleave C5 twice (figure 6). After much research we learned that while no one has ever described the ability of PSA to cleave any immune proteins, particularly complement proteins, other proteases had be shown to cleave C3, another protein integral to the complement system. The two proteases shown to cleave C3 were membrane type-1 matrix metalloproteinase [5] and procathepsin-L [6], both associated with the progression of cancers. We used BLAST [7] to compare sequence similarity between C3 and C5 and learned the two proteins were very similar, with 48% of the residues being "positives". It is well known that alpha-2-macroglobulin, C3, C4, and C5 all evolved from a common ancestor, so the sequence similarity is to be expected. To test whether this phenomenon was unique to C5 or if it was shared with complement proteins C3 and C4, we repeated the PSA-mediated cleavage experiment with C3 and C4.

It was shown that PSA also had the ability to cleave C3 (figure 7), but not C4 (data not shown). Could this PSA-specific cleavage of complement proteins C3 and C5 be the serendipitous discovery of a previously unrecognized immunomodulatory function of PSA? We chose to interrogate this further by characterizing the

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Figure 7: Results of PSA mediated wild-type C3 cleavage, analyzed by gel electrophoresis and protein staining. C3 was added to enzymatically active PSA in buffer or buffer alone. Time points were taken at 0 hour, 1 hour, 3 hours, and overnight. In the absence of PSA, C3 seems to be stable in buffer as no significant shifts were observed. However, when C5 is treated with PSA the 113kDa alpha chain appears to be cleaved twice.
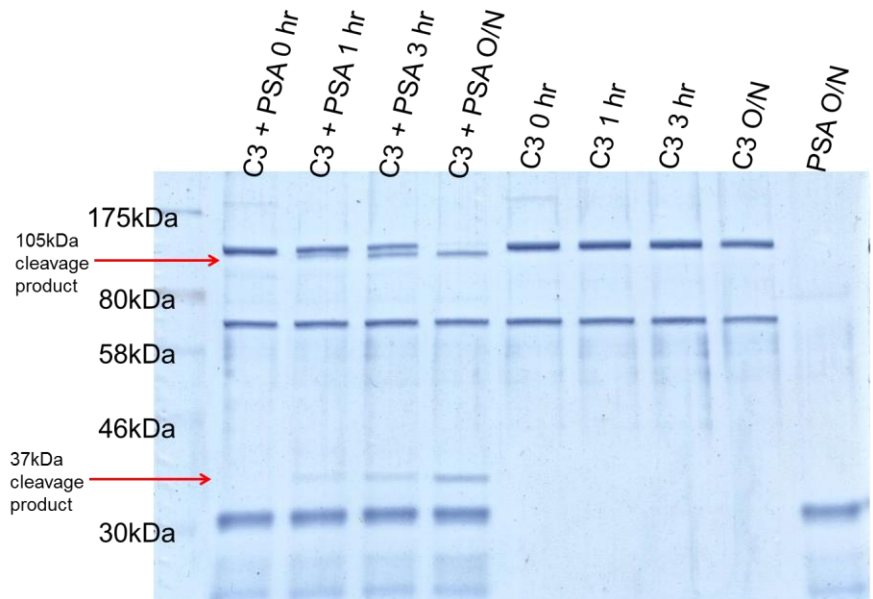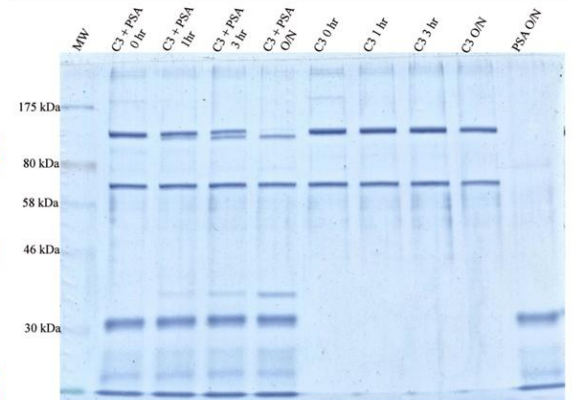
Figure 8: Results of N-terminal sequencing of PSA-generated C3 cleavage products. We chose to sequence the 113kDa, 105kDa, and 37kDa bands to gain a full understanding of C3 after PSA-mediated cleavage. Our results surprisingly indicate PSA has both trypsin-like and chymotrypsin-like activity. Cleavage sites are represented by "*//*".

sites of PSA-mediated cleavage. This would serve two purposes: first, it would be necessary to know the sites of proteolysis so we could mutate them and make them non-substrates of PSA. This would allow us to go further with the production of PAC-2; however, we would have to backtrack and make a new wild-type PSA-resistant C5, then substitute in the same PSA recognition site from PAC-2. Characterizing the sites of proteolysis would also be helpful in understanding whether the newfound ability of PSA to cleave C3 and C5 might just be an in vitro artifact or have biological meaning.

We chose to use N-terminal sequencing (Edman degradation) to reveal the sites of PSA-mediated proteolysis. As before, C3 and C5 were treated overnight with enzymatically active PSA. The reactions were separated by SDS-PAGE, then transferred to PVDF membrane by wet transfer. The membrane was then stained with coomassie blue. For C3, we chose to sequence the 113kDa, 105kDa, and 37kDa bands. The bands were excised and sent to the Johns Hopkins Synthesis & Sequencing Facility. Results indicate PSA has both trypsin-like and chymotrypsin-like activity (figure 8). This is confusing as it is known PSA is a chymotrypsin-like serine protease. However, in the past it had been reported that preparations of PSA were contaminated with hK2[8]. hK2 is serine protease that is closely related of PSA (hK3), with the major difference being sequence specificity - hK2 is trypsin-like, while hK3 is chymotrypsin-like. They are both found in the seminal plasma, although at different levels, with PSA being approximately 100X more abundant. Commercially available PSA, like that used in our lab, is purified from seminal plasma. It is likely that our PSA is contaminated with hK2 or another trypsin-like protease. This may or may not be an issue in future experiments, given certain controls are implemented.

Specific Aim 4 – Inject tumor bearing mice with PAC5 and measure tumor regression. [Not yet begun]

Key Research and Training Accomplishments:

- With the help of computer modeling a series of C5 mutants were made that led to the discovery of PAC-2, a PSA cleavable C5 as verified by mass spectroscopy.
- An adenovirus expressing PAC-2 has been made to scale up recombinant production of the PSA cleavable C5.
- PAC-2 was purified from conditioned media of virally-transfected cells.
- Enzymatic treatment of PAC-2 with PSA revealed a curious cleavage pattern.
- We learned even wild-type C5 is a substrate of PSA; subsequently we learned C3 is also a substrate, but not C4.
- Characterized the sites of PSA-mediated C3 proteolysis.
- The PI has written his thesis proposal, organized his thesis committee, and held five committee meetings, gave three poster presentations at national meetings, and two oral presentations at Johns Hopkins.

Reportable Outcomes:

The discovery of PAC-2 was central to funding we applied for in June 2009 and subsequently received (W81XWH-10-PCRP-IDA). The PI presented a poster detailing the results herein at the Johns Hopkins Prostate Research Day (February 5th 2011), the IMPaCT meeting (March 10, 2011), and Multi-Institutional Prostate Cancer Program Retreat (March 22nd 2011).

Conclusion:

We have made substantial progress in fulfilling the goals outlined in our original statement of work. We hope to characterize the sites PSA mediated C5 cleavage in the coming weeks, which will give us the information needed to re-engineer PAC-2 so it is selectively activateable. We also hope to better understand the

biological relevance, if any, related to the PSA mediated cleavage of complement proteins C3 and C5. I am up to date on all institutionally mandated training requirements.

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Appendices: (none)